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(54) Title: GROWTH HORMONE SECRETAGOGUE RELATED RECEPTORS AND NUCLEIC ACIDS (57) Abstract This invention relates to a new family of receptors, growth hormone secretagogue-related receptors, which exhibit moderate sequence identity to both the growth hormone secretagogue receptor (GHS-R) and the neurotensin receptor (NT-R). These newly identified receptors are expressed in a diverse set of tissues. This invention also relates to nucleic acids encoding these receptors, and to the use of these receptors to identify ligands that modulate growth hormone release or other endocrine functions.		

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TITLE OF THE INVENTION

Growth Hormone Secretagogue Related Receptors and Nucleic Acids

FIELD OF THE INVENTION

- 5 This invention relates to a new family of receptors, growth hormone secretagogue-related receptors (GHSR-Rs), nucleic acids encoding these receptors, and to the use of these receptors to identify ligands that modulate GHSR-R function.

BACKGROUND OF THE INVENTION

- 10 Growth hormone secretagogues (GHSs) and secretagogue-like compounds, both peptide and non-peptide, bind to and exert their biological effects (i.e., release of growth hormone (GH)) through a G protein-coupled receptor (GPC-R) distinct from the receptors for growth hormone releasing hormone (GHRH) and somatostatin (SST) (Pong et al., 1996 *Mol. Endocrin.* 10:57-61). The molecular
15 cloning of the growth hormone secretagogue receptor (GHS-R) capitalized on the pivotal observation that GHSs transduce their signal through activation of the phospholipase C pathway (Cheng et al., 1991 *Endocrinology* 129:3337-3342; Howard et al., 1996 *Science* 273:974-977). cDNA and genomic DNA cloning from human, swine, and rat showed that the GHS-R is a remarkably conserved protein of 364/366
20 amino acids containing 7 putative alpha-helical transmembrane (TM) domains, a signature feature of GPC-R's (Howard et al. 1996; McKee et al., 1997 *Mol. Endocrin.* 11:415-423). In all species evaluated, the GHS-R is encoded by a single highly-conserved gene containing one intron, placed at the C-terminal end of TM domain 5. The GHS-R exhibits the highest sequence similarity to the receptors for neurotensin
25 (NT-R) with sequence identity of 34%. The biology of the growth hormone secretagogues (GHSs) is still in a relatively early stage of development. Research is focused on identification of the GHS natural ligand system and understanding the role of the GHS-R in brain regions (substantia nigra, dentate gyrus, hippocampus) other than those traditionally thought to be involved in GH secretion (Bennett et al. 1997;
30 Guan et al. 1997).

- The identification of other G-protein coupled receptors points to the existence of a new natural ligand pathway perhaps divergent from the neuropeptide neurotensin and the GHS natural ligand. Two new human full-length GPC-R's entitled GPR38 and GPR39 were cloned having 52 % and 32 % protein sequence
35 identity to the human GHS-R, respectively (McKee et al. 1997).

It would be desirable to identify other GPC-Rs perhaps impactful on GH release and elucidate their functions. It would also be desirable to identify ligands particular to these receptors that play an important role in these and other associated pathways.

5 One peptide for which receptors have not been molecularly identified to date is neuromedin U. Neuromedin U (NMU) is a neuropeptide first isolated from porcine spinal cord in two molecular forms, one containing 25 amino acids (NMU-25) and the other one 8 amino acids (NMU-8); Minamino *et al.*, 1985 *Biochem Biophys Res Commun* 130:1078-85. It was subsequently isolated from rat (NMU-23),
10 human (NMU-25), frog (NMU-25), dog (NMU-8 and NMU-25), rabbit (NMU-25), and chicken (NMU-25); Domin *et al.*, 1986 *Biochem Biophys Res Commun* 140:1127-34; Conlon *et al.*, 1988 *J Neurochem* 51:988-91; Minamino *et al.*, 1988 *Biochem Biophys Res Commun* 156:355-60; Domin *et al.*, 1989 *J Biol Chem* 264:20881-5; O'Harte *et al.*, 1991 *Peptides* 12:11-5; Kage *et al.*, 1991 *Regul Pept* 33:191-8; and Domin *et al.*, 1991 *Regul Pept* 41:1-8. Mammalian NMUs share a
15 common C-terminal sequence-Phe-Leu-Phe-Arg-Pro-Arg-Asn-amide which appears to be essential for its biological activities. NMU is distributed both in the gastrointestinal tract and the central nervous system (CNS).

In the rat, the highest concentration of NMU was found in the ileum,
20 followed by the pituitary, hypothalamus, spinal cord, thyroid, and the genitourinary tract. Immunohistochemistry studies showed that NMU immunoreactivity in the gut was only found in nerve fibers, mainly in the myenteric and submucous plexuses, and in the mucosa of all areas except stomach while no NMU immunoreactivity was found in endocrine cells. In the rat brain, NMU immunoreactivity was found in fibers
25 widespread throughout the brain with the exception of the cerebellum.

Human and rat genes encoding NMU precursor have been isolated. Both encode NMU at the C-terminus and other potential peptide products in the middle; Lo *et al.*, 1992 *Mol Endocrinol* 6:1538-44; Austin *et al.*, 1995 *J Mol Endocrinol* 14:157-69.

30 High affinity NMU binding was characterized in rat uterus, and was shown to be sensitive to GTP- γ -S (Nandha *et al.*, 1993 *Endocrinology* 133:482-6), suggesting the receptor for NMU was a G-protein coupled receptor. However, no receptor of NMU has been molecularly identified so far.

The physiological role of NMU remains largely unrecognized. It can
35 cause potent contraction of smooth muscle, increase arterial blood pressure, modify

intestinal ion transport, and at low doses stimulates the function and growth of the adrenal cortex. NMU was also shown to reduce the blood flow in superior enteric artery and portal vein while increase blood flow slightly in pancreatic tissue. Nevertheless, NMU is the only neuromedin without a receptor cloned nor a great deal of knowledge obtained concerning its pharmacology and physiology.

It would be most desirable to identify a G-protein coupled receptor responsive to neuromedin U or ligands sufficiently similar thereto. A receptor responsive to neuromedin U would greatly facilitate our understanding of the physiological mechanisms of neuromedin U and other ligands sufficiently similar thereto.

SUMMARY OF THE INVENTION

This invention relates to a new family of G protein-coupled receptors, growth hormone secretagogue-related receptors (GHSR-Rs) free from receptor-associated proteins, which exhibit moderate protein sequence identity (33 and 29 %) to both the growth hormone secretagogue receptor (GHS-R) and the neurotensin receptor (NT-R) type 1, respectively. Particularly, the full-length mouse and human GHSR-Rs have been identified. These newly identified receptors are expressed in a diverse set of tissues. A further aspect of this invention is the above receptors which are isolated or purified.

Another aspect of this invention are GHSR-Rs which are encoded by substantially the same nucleic acid sequence, but which have undergone changes in splicing or other RNA processing-derived modifications or mutagenesis induced changes, so that the expressed protein has a homologous, but different amino acid sequence from the native form. These variant forms may have different and/or additional functions in animal physiology or *in vitro* in cell based assays.

Growth hormone secretagogue related receptors (GHSR-Rs) are proteins containing various functional domains, including one or more domains which anchor the receptor in the cell membrane, and at least one ligand binding domain. As with many receptor proteins, it is possible to modify (e.g., by deletion) many of the amino acids, particularly those which are not found in the ligand binding domain, and still retain at least a percentage of the biological activity of the original receptor. This invention specifically includes such

modified functionally equivalent GHSR-Rs as well as receptors comprising the binding domain of a GHSR-R of this invention.

Additionally, it is possible to modify other functional domains such as those that interact with second messenger effector systems, by altering binding
5 specificity and/or selectivity. Such functionally equivalent mutant receptors are also within the scope of this invention.

Another aspect of this invention are nucleic acids which encode growth hormone secretagogue related receptors (GHSR-Rs). More specifically, the invention relates to nucleic acids comprising the sequences of SEQ ID NOs: 1
10 and 3 as well as those which hybridize to same under highly stringent conditions. These nucleic acids may be free from associated nucleic acids, or they may be isolated or purified. For most cloning purposes, cDNA is a preferred nucleic acid, but this invention specifically includes other forms of DNA as well as RNAs which encode a GHSR-R.

Yet another aspect of this invention relates to vectors which
15 comprise nucleic acids encoding a GHSR-R. These vectors may be comprised of DNA or RNA; for most cloning purposes DNA vectors are preferred. Typical vectors include plasmids, modified viruses, bacteriophage and cosmids, yeast artificial chromosomes and other forms of episomal or integrated DNA that can
20 encode a GHSR-R. It is well within the skill of the ordinary artisan to determine an appropriate vector for a particular gene transfer or other use.

A further aspect of this invention are host cells which are transformed with a gene which encodes a growth hormone secretagogue related receptor. The host cell may or may not naturally express a GHSR-R on the cell
25 membrane. Preferably, once transformed, the host cells are able to express a growth hormone secretagogue related receptor on the cell membrane. Depending on the host cell, it may be desirable to adapt the DNA so that particular codons are used in order to optimize expression. Such adaptations are known in the art, and these nucleic acids are also included within the scope of this invention.
30 Generally, mammalian cell lines, such as COS, HEK-293, CHO, HeLa, NS/O, CV-1, GC, GH3 or VERO cells are preferred, but other cells and cell lines such as *Xenopus* oocytes or insect cells, may also be used. Both cell lines transformed to express the GHSR-R receptor and those naturally expressing the receptor are included for use within the following assays.

One further aspect of this invention is a method of identifying ligands comprising contacting cells expressing the GHSR-R receptor in accordance with the instant invention with a compound suspected of being a ligand specific for said receptor and determining whether binding occurs, binding
5 constituting a positive indication of the presence of a ligand.

Another aspect of this invention is a method of identifying ligands for GHSR-R which comprises contacting cells expressing the GHSR-R receptor with a compound suspected of being a ligand specific for said receptor in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+}
10 mobilization, and monitoring for luminescence or other signal from the reporter indicating activation of the receptor, activation constituting a positive indication of the presence of a ligand.

Another aspect of the instant invention is a method of identifying ligands for GHSR-R which comprises contacting cells expressing the GHSR-R
15 receptor with a compound suspected of being a ligand specific for said receptor, and monitoring for changes in concentration of intracellular cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of the presence of a ligand.

An additional aspect of the invention is a method for determining whether a substance is a potential agonist or antagonist of GHSR-R comprising
20 contacting cells expressing the GHSR-R receptor with labeled neuromedin U in the presence and in the absence of the substance, and measuring the binding of neuromedin U to GHSR-R, where if the amount of binding of neuromedin U is more or less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GHSR-R, respectively.

25 A further aspect of the instant invention is a method of determining whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor with the substance in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the
30 receptor; activation constituting a positive indication of the presence of an agonist.

Another aspect of the instant invention is a method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor first with the substance and then with
35 neuromedin U in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter

indicating activation of the receptor; where if the amount of luminescence or signal is less in the presence of the substance than in the absence of the substance, then the substance is a potential antagonist of GHSR-R.

Another aspect of the instant invention is a method of determining
5 whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor with the substance, and monitoring for changes in cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of an agonist.

Another aspect of the instant invention is a method of determining
10 whether a substance is a potential antagonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor with the substance, and monitoring for changes in cyclic AMP (cAMP); a marginal to no increase in cAMP constituting a positive indication of an antagonist.

Another aspect of the instant invention is a method for the treatment or
15 prevention of obesity which comprises administering to a mammal in need of such treatment or prevention an effective amount of neuromedin U or a GHSR-R agonist.

A further aspect of the instant invention is a method of decreasing food intake of a mammal which comprises administering to said mammal an effective amount of neuromedin U or a GHSR-R agonist.

Yet one further aspect of this invention is a method of determining
20 whether a compound binds to both growth hormone secretagogue receptor (GHS-R) and growth hormone secretagogue related receptor (GHSR-R) which comprises contacting both GHS-R and GHSR-R with the compound or ligand of interest and determining whether binding occurs to both receptors.

25

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

FIGURE 1A-C presents the DNA (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences for mouse GHSR-R. Putative transmembrane alpha helices are overlined and numbered from 1 to 7.

30 FIGURE 2A-C shows the DNA (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequences for human GHSR-R. Putative transmembrane alpha helices are overlined and numbered from 1 to 7.

FIGURE 3A-C presents a comparison of the GHSR-R of the present invention to other members of the GHS-R/NT-R Family.

35 FIGURE 4A is a Northern Blot Analysis of GHSR-R expression.

FIGURE 4B is a Southern Blot Analysis of GHSR-R expression.

FIGURE 5 illustrates activation of GHSR-R by NMU by the aequorin assay. HEK293/aeq cells stably expressing GHSR-R (solid symbols) and untransfected cells (open symbols) were assayed (n=3) against 5-fold serial dilutions of rat NMU-23 (circles) and porcine NMU-8 (squares).

FIGURE 6 illustrates activation of GHSR-R by NMU assayed with FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices, Inc.). Cos-7 cells were transiently transfected with GHSR-R/pIRESpuromycin and control vector and assayed versus rat NMU-23 (triangles) and porcine NMU-8 (circles) 72 hours later (n=6).

FIGURE 7 is a radioligand binding assay of NMU. Competition for 125I-labeled rat NMU-23 was performed using HEK293/aeq cells stably expressing GHSR-R.

FIGURE 8 illustrates the dose response of GHSR-R to NMU in CHO-NFAT-bla cells by β -lactamase assay. NMU was diluted by 10-fold serial dilutions and assayed in triplicates. The EC₅₀ of NMU for GHSR-R in this assay is ~75 pM.

FIGURE 9 illustrates the dose response of GHSR-R to NMU in HEK293CRE-bla cells by β -lactamase assay. NMU was diluted by 10-fold serial dilutions and assayed in triplicates. The EC₅₀ of NMU for GHSR-R is about 3 nM in this assay.

DETAILED DESCRIPTION OF THE INVENTION

As used throughout the specification and claims, the following definitions shall apply:

“Growth hormone secretagogue related receptor” or “GHSR-R” or “FM-3” includes an amino acid sequence encoded by a nucleic acid molecule or fragment thereof that (a) comprises the nucleotide sequence as set forth in SEQ ID NOs:1 or 3; (b) comprises a nucleic acid sequence encoding a polypeptide that is at least 70 percent identical, preferably at least 80 percent identical and most preferably at least 90 percent identical to the polypeptide encoded by SEQ ID NOs:2 or 4; (c) is a naturally occurring allelic variant of (a) or (b); (d) is a nucleic acid variant of (a)-(c); and/or (e) is complementary to (a)-(d). This term also includes peptide or polypeptide fragments derived from items (a)-(e) above, to the amino acid sequences set forth in SEQ ID NOs:2 or 4 and/or to chemically modified derivatives as well as nucleic acid and/or amino acid sequence variants thereof.

"GHSR-R derivative" or "GHSR-R variant" refers to a GHSR-R that has (1) been chemically modified, as for example, by addition of polyethylene glycol or other compound, and/or (2) contains one or more nucleic acid or amino acid sequence substitutions, deletions and/or insertions.

5 "Nucleic acid variant" refers to a sequence wherein one or more nucleotides have been designed to differ from the sequence at issue (in this case, GHSR-R).

"Stringent conditions" refers to such hybridization reaction parameters as the concentration and type of ionic species present in the hybridization solution, the
10 types and concentrations of denaturing agents present, and the temperature of hybridization. These conditions are approximately 35°C to 65°C in a salt solution of approximately 0.015 to 0.9 molar NaCl. Generally as hybridization conditions become highly stringent (such as 0.1 x SSC, 65°C), longer probes are preferred if stable hybrids are to be formed. As a rule, the stringency of the conditions under
15 which a hybridization is to take place will dictate certain characteristics of the preferred probes to be employed. Such relationships are well understood and can be readily manipulated by those skilled in the art.

"Binding domain" refers to that region or regions of the receptor which are able to bind to ligands.

20 "Ligand" refers to any molecule which binds to the GHSR-Rs of this invention. These ligands can have either agonist, partial agonist, partial antagonist or antagonist activity.

"Free from receptor-associated proteins" means the receptor protein is not in a mixture or solution with other membrane receptor proteins.

25 "Free from associated nucleic acids" means the nucleic acid is not covalently linked to DNA which it is naturally covalently linked in the organism's chromosome.

"Isolated receptor" means the protein is not in a mixture or solution with any other proteins.

30 "Isolated nucleic acid" means the nucleic acid is not in a mixture or solution with any other nucleic acid.

"Purified receptor" means the receptor is at least about 95% pure.

"Purified nucleic acid" means the nucleic acid is at least about 95% pure.

“Secretagogue-like compound” means any compound which exhibits substantially the same function as growth hormone secretagogues (i.e., stimulating growth hormone release).

“NMU” means neuromedin U.

5 “Ligand(s) sufficiently similar thereto” means any ligand which binds to the GHSR-R and causes some response.

A “GHSR-R agonist” is a compound which binds to GHSR-R and produces a cellular response which is at least about equivalent to that of neuromedin U and which may be greater.

10 A “GHSR-R antagonist” is a compound which binds to GHSR-R and produces a cellular response which is less pronounced than that of neuromedin U.

“Activation” means that the receptor is stimulated to carry out its normal function, herein indicated by release of the second messenger Ca^{2+} .

15 “Effective amount” means an amount effective in bringing about the intended result (i.e., decrease in food intake or treatment of obesity). Preferably, this dose is to be above 1 μg of neuromedin U or agonist thereof/kg body weight, more preferably, above 10 $\mu\text{g}/\text{kg}$, and most preferably, above 20 $\mu\text{g}/\text{kg}$.

The present invention relates to newly identified receptors, growth hormone secretagogue related receptors (GHSR-Rs), which exhibit moderate
20 sequence identity to the growth hormone secretagogue receptor (GHS-R) and the neurotensin receptor (NT-R). More specifically, this invention relates to receptors comprising the sequence of SEQ ID NOs:2 or 4, the mouse and human receptor sequences, respectively. The human and murine GHSR-Rs exhibit strong protein sequence identity (73%). This invention also relates to proteins that are at least
25 90% homologous to said receptors. Receptors in accordance with this invention group as a separate branch distinct from both GHS-R and NT-R sequences and the orphan GPC-R's GPR38 and GPR39; see Figure 2.

The receptors of this invention have been proven by Applicants to be high affinity receptors of neuromedin U (NMU), a neuropeptide widely distributed in
30 the gut and central nervous system which causes potent contraction of rat uterine smooth muscle. The discovery of the first NMU receptor, designated GHSR-R, provides important information for understanding the biochemical mechanisms and physiological roles of NMU.

HEK293 cells and Cos-7 cells transfected with human GHSR-R
35 showed strong, dose-dependent calcium mobilization in response to both the long

form (rat NMU-23) and the short form (porcine NMU-8) of neuromedin U. Radioligand binding analysis showed high affinity binding of NMU ($IC_{50} = 3 \text{ nM}$) to membrane preparations isolated from HEK293 cells stably expressing human GHSR-R. Further, CHO-NFAT-bla cells stably expressing GHSR-R showed a strong
5 response to NMU when screened against NMU using the β -lactamase assay of Example 8. The following studies suggest GHSR-R most likely couples to the G_q/G_{11} pathway since activation leads to strong calcium mobilization. Both rat NMU-23 and porcine NMU-8 were able to activate human GHSR-R with nanomolar affinity.

10 The mouse GHSR-R receptor sequence contains a single intron of approximately 3 kb at bp 878 (a perfectly conserved splice donor site: G/gt) interrupting the open reading frame sequence at amino acid Asp²⁹³. This immediately follows the predicted TM domain 6 (exon 1: extracellular domain through TM-6; exon 2: third extracellular loop, TM-7 and C-terminal intracellular
15 domain). Without being bound by theory, it is believed that a non-canonical leucine codon (Soldata et al. 1990 *J. Biol. Chem.* 265:4498-4506) serves as the initiator based on sequence similarity to the human sequence in this region and the absence of upstream in-frame methionine codons for an additional 308 basepairs.

By contrast, the human genomic sequence contains an initiator
20 methionine codon in a favorable Kosak context (Kosak, M. 1984 *Nucleic acids res.* 12:857-872) with an in-frame stop codon present 102 bp upstream.

Both forms contain all the hallmark features of the 7-transmembrane domain (TM)-containing G-protein linked receptor superfamily (GPC-Rs or 7-TM receptors). These include the seven transmembrane regions, three intra- and
25 extracellular loops, and the GPC-R protein signature sequence. Thus, GHSR-Rs, and specifically the sequences of SEQ ID Nos:2 and 4, constitute a new member of the GPC-R family of receptors. Not all regions are required for functioning, and therefore this invention also comprises functional receptors which lack one or more non-essential domains.

30 An expression profile derived from a Northern blot analysis revealed a predominate transcript size of approximately 5 kb, which was detected in all tissues examined. A band of approximately 2 kb was also noted in most of the tissues (with high abundance in testis), suggesting alternative mRNA processing of the GHSR-R primary transcript.

Southern blot analysis (Fig. 3B) of EcoR1-digested genomic DNA using the mouse form as a radiolabeled probe gave a simple hybridization pattern in all species tested, indicative of a single, highly conserved gene encoding GHSR-R.

It is to be noted that the GHSR-Rs and fragments thereof are
5 immunogenic. Thus, another aspect of this invention is antibodies and antibody fragments which can bind to GHSR-R or a GHSR-R fragment. These antibodies may be monoclonal antibodies and produced using either hybridoma technology or recombinant methods. They may be used as part of assay systems or to deduce the function of a GHSR-R present on a cell membrane.

10 Another aspect of this invention are antisense oligonucleotides, nucleotides which can bind to GHSR-R nucleotides and modulate receptor function or expression.

Yet another aspect of this invention is a method of increasing the amount of GHSR-Rs on a cell membrane comprising, introducing into the cell a
15 nucleic acid encoding a GHSR-R, and allowing expression of the GHSR-R.

A further aspect of this invention is a method of identifying ligands comprising contacting cells expressing GHSR-R with a compound suspected of being a ligand for said receptor and determining whether binding occurs, binding constituting a positive indication of the presence of a ligand. Ligands detected
20 using the assays described herein may be used as modulators of endocrine function. Further, ligands capable of mimicking the functions of neuromedin U could be identified via this method.

Another aspect of this invention is a method of identifying ligands for GHSR-R which comprises contacting cells expressing the GHSR-R receptor
25 with a compound suspected of being a ligand specific for said receptor in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} mobilization, and monitoring for luminescence or other signal from the reporter indicating activation of the receptor, activation constituting a positive indication of the presence of a ligand. The aequorin assay is a sensitive method to measure
30 Ca^{2+} mobilization based on bioluminescence of jelly fish aequorin in the presence of Ca^{2+} ; Button and Brownstein, 1993 *Cell Calcium* 14:663-671. Other suitable assay systems responsive to Ca^{2+} mobilization include the use of various fluorescent dyes that monitor Ca^{2+} concentration change (Kao et al., 1989, *J. Biol. Chem.*, 264:8179-8184), and the use of transcription-based reporter systems that

monitor changes of calcineurin activity (Zlokarnik et al., 1998, *Science*, 279:84-88).

Another aspect of the instant invention is a method of identifying ligands for GHSR-R which comprises contacting cells expressing the GHSR-R receptor with a compound suspected of being a ligand specific for said receptor, and monitoring for changes in concentration of intracellular cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of the presence of a ligand. Suitable assay systems capable of monitoring cAMP changes include direct measurement of intracellular concentrations of cAMP by ELISA and the use of transcription-based reporter assays that are responsive to cAMP changes.

An additional aspect of the invention is a method for determining whether a substance is a potential agonist or antagonist of GHSR-R comprising contacting cells expressing the GHSR-R receptor with labeled neuromedin U in the presence and in the absence of the substance, and measuring the binding of neuromedin U to GHSR-R, where if the amount of binding of neuromedin U is more or less in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GHSR-R, respectively. Agonists are useful in the treatment, control, or prevention of diseases, disorders or conditions responsive to the activation of the neuromedin U receptor. As such, agonists could be useful in the treatment, control or prevention of depression, anxiety, compulsion, neuroses, insomnia/sleep disorders, substance abuse, pain, neuroprotective and cognitive disorders, and memory enhancement including the treatment of Alzheimer's disease.

A further aspect of the instant invention is a method of determining whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor with the substance in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the receptor; activation constituting a positive indication of the presence of an agonist. The aequorin assay is a sensitive method to measure Ca^{2+} mobilization based on bioluminescence of jelly fish aequorin in the presence of Ca^{2+} ; Button and Brownstein, 1993 *Cell Calcium* 14:663-671. Other suitable assay systems responsive to Ca^{2+} mobilization include the use of various fluorescent dyes that monitor Ca^{2+} concentration change (Kao et al., 1989, *J. Biol. Chem.*, 264:8179-

8184), and the use of transcription-based reporter systems that monitors changes of calcineurin activity (Zlokarnik et al., 1998, *Science*, 279:84-88).

Another aspect of the instant invention is a method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting
5 cells expressing the GHSR-R receptor first with the substance and then with neuromedin U in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the receptor; where if the amount of luminescence or signal is less in the presence of the substance than in the absence of the substance, then the
10 substance is a potential antagonist of GHSR-R.

Another aspect of the instant invention is a method of determining whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the GHSR-R receptor with the substance, and monitoring for changes in cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of an
15 agonist. Assay systems capable of monitoring cAMP changes include direct measurement of intracellular concentrations of cAMP by ELISA, and the use of transcription-based reporter assays that are responsive to cAMP changes.

Another aspect of the instant invention is a method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting
20 cells expressing the GHSR-R receptor with the substance, and monitoring for changes in cyclic AMP (cAMP); a marginal (non-biologically significant) to no increase in cAMP constituting a positive indication of an antagonist.

A further aspect of the instant invention is a method of decreasing food intake of a mammal which comprises administering to said mammal an effective
25 amount of neuromedin U or a GHSR-R agonist. Applicants were the first to identify that neuromedin U could be used in the regulation of food intake. Upon administration of neuromedin U to rats, there was a suppression of food intake. Accordingly, selectively modulating GHSR-R receptor signaling may be an approach to treatment of human obesity and other eating disorders. Obesity, a condition defined
30 as being 20% over one's ideal body weight, is a serious public health concern in the industrialized world. According to the National Institutes of Health, over 97 million Americans are overweight or obese. Obesity predisposes individuals to potentially life-threatening conditions such as hypertension, stroke, heart disease and diabetes and is the second leading cause of preventable deaths in the US. Identification of
35 weight-regulating therapeutics (agonists) that modulate the GHSR-R receptor may

Moreover, cachexia (malnutrition) is estimated to be responsible for approximately 40% of all cancer deaths. Accordingly, therapeutics inducing weight gain, such as antagonists of the GHSR-R gene could serve an important area of medical need. In this respect, a method included within the instant invention would be a method of increasing food intake of a mammal which comprises administering to said mammal an effective amount of a GHSR-R antagonist.

Yet one further aspect of the present invention is a method by which to determine whether a compound of interest binds to both GHSR-R and GHS-R. This is useful for identifying compounds with very specific binding characteristics (e.g., those that only bind one receptor as opposed to the other) which would be helpful when a very specific ligand is needed as well as for identifying those compounds which perhaps have a complementary or, adversely, an antagonistic effect in binding both receptors.

25 EXAMPLE 1

score ($P(N) 1.3 \times 10^{-12}$) to the human GHS-R TM domains 6-7 (residues 265-366). Upon further analysis, EST 562357 exhibited good sequence identity (63 % DNA, 36 % amino acid) to the 3' end of the gene for the human GHS-R. A murine T cell LXR cDNA library (Stratagene) was screened with the mouse EST 562357 probe (455 bp in length) under high-stringency conditions. Four partial clones were identified after three rounds of screening. Additional clones were isolated from mouse thymus poly

(A)⁺ RNA via 5' Race Marathon cDNA Amplification (Clontech). PCR of the IXR cDNA library with gene specific primers and the library adaptor primer resulted in the isolation of a full-length clone. Screening of a lFixII mouse genomic library (Stratagene) identified eight positive clones to aid in the determination of the correct start codon.

EXAMPLE 2

Isolation of the Human GHSR-R

To isolate the human isoform, a human PAC library (Genome Systems) was hybridized (32°C in 50% formamide, 5X SSPE) and washed at moderate stringency (55°C, 1 X SSC) with a probe derived from the ORF of the mouse isoform. Two positive clones were identified. The PAC clones were isolated and subjected to restriction enzyme digestion and Southern blotting providing a BamH1 fragment of approximately 5 kb containing the complete ORF for the human isoform. DNA was sequenced on both strands using dye terminator cycle sequencing ready reactions (Perkin Elmer-ABI), and analyzed on a 377 ABI Prism cycle sequencer.

EXAMPLE 3

Comparison of GHSR-R to other members of the GHS-R/NT-R Family

Protein sequences were aligned using the Pileup program (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI.; gap extension 4, gap creation 12); results indicated in FIGURE 2. Identical residues are boxed. The sequences used in the alignment and their Genbank database accession numbers are: human GHS-R (HSU60179); human NT-R type 1 (X70070); human NT-R type 2 (2494989); human GPR38 (AF034632); human GPR39 (AF034633).

EXAMPLE 4

Expression Profile via Northern Blot Analysis

A commercial RNA blot (Clontech) containing poly (A)⁺ mRNA (1 µg/lane) from several mouse tissues was hybridized with a radiolabeled probe

encompassing the mouse GHSR-R ORF. Following high-stringency post-hybridizational washing, the blot was exposed to X-ray film for four days at -70°C. RNA size marker (Life Technologies) are, in kb, 9.5, 7.5, 4.4, 2.4, and 1.35.

The predominate transcript size was approximately 5 kb, which was detected in all tissues examined. A band of approximately 2 kb was also noted in most of the tissues (with high abundance in testis), suggesting alternative mRNA processing of the GHSR-R primary transcript.

EXAMPLE 5

10

Expression Profile via Southern Blot Analysis

A genomic Southern blot (EcoRI-digested DNA, 10 mg/lane) was hybridized with a 3' fragment of the human GHSR-R ORF (third intracellular loop to C-terminal intracellular domain). Post-hybridizational washing stringencies were at 55°C, 4 X SSPE after which the filters were dried and exposed to X-ray film for 5 days at -70°C. Lambda Hind III DNA markers were (in kb), 23.1, 9.4, 6.6, 4.4, 2.3, 2.1.

Using the mouse form as a radiolabeled probe, the results revealed a simple hybridization pattern in all species tested, indicative of a single, highly conserved gene encoding GHSR-R.

EXAMPLE 6

Construction of GHSR-R-Expressing Plasmid

The complete coding sequence of human GHSR-R was amplified by PCR using plasmid containing human GHSR-R as template with two primers, the forward primer, FM3EcoRV.F, 5'-CTGAGATATCACCACCATGGCTTGCAATGGCAGTGC-3' and the reverse primer, hFM3BamHI.R, 5'-AGTCGGATCCGTATCAGGATGGATCGGTCTCTTGCT. The forward primer contained an EcoRV site and consensus Kozak (ACCACC) sequence for translation immediately upstream of the initiation codon. The reverse primer contained an BAMHI site downstream of the stop codon.

PCR reactions were carried out using the DNA polymerase PFU turbo (Stratagene, La Jolla, CA, USA) following the conditions of the enzyme supplier. The PCR product was purified, digested by EcoRV plus BamHI, and ligated into the

vector pIRESpuromycin (Clontech, Palo Alto, CA, USA) which was also digested with EcoRV and BamHI using T4 DNA ligase. The ligation product was transformed into E.coli. Clones containing the correct construct were identified by restriction digestion and verified by DNA sequencing. The resulting plasmid is called GHSR-R-pIRESpuro.

EXAMPLE 7

Generation of GHSR-R-expressing cells

Plasmid DNA of GHSR-R-pIRESpuro was linearized by digestion with FspI and transfected into CHO-NFAT-bla cells and HEK293CRE-bla cells (Aurora Biosciences, San Diego, CA, USA), and HEK293aequorin cells using lipofectamine (GIBCO-BRL, Gaithersburg, MD, USA) by following the conditions suggested by GIBCO-BRL. Three days after transfection, the cells were detached by trypsin digestion, diluted by 1:5 in complete culture medium plus puromycin at 5.0 µg/ml for CHO-NFAT-bla cells, or 0.5 µg/ml for both HEK293CRE-bla cells and HEK293aequorin cells. Cells were incubated at 37 °C/5% CO₂ and replaced with fresh medium twice per week. Two weeks after transfection, puromycin-resistant cells were detached by trypsin digestion, combined, and propagated for screening.

EXAMPLE 8

Aequorin Bioluminescence and β-lactamase assays

Aequorin bioluminescence assays were carried out following the protocol of Button and Brownstein (1993 *Cell Calcium* 14:663-71) with minor modification. ECB (extracellular buffer) was replaced by Ham's F12 medium (with 0.3 mM CaCl₂, 0.1% fetal bovine serum, 25 mM HEPES, pH7.3).

HEK293aeq/17 cells stably expressing GHSR-R were maintained at 37°C /5% CO₂ in DMEM + 10% fetal bovine serum (heat inactivated), 1 mM sodium pyruvate, 500 µg/ml neomycin G418, 0.5 µg/ml puromycin (GIBCO-BRL, Gaithersburg, MD, USA). Cells were seeded by 1:4 dilution in T75 flasks two days before assay. The day the assay was performed, cells at 80-90% confluency were washed twice with DMEM + 0.1 % fetal bovine serum, and then charged for one hour at 37 °C /5% CO₂ in DMEM containing 8 µM coelenterazine cp (Molecular Probes, Eugene, OR, USA) and 30 uM glutathione. Cells were then washed once with

versene (GIBCO-BRL, Gaithersburg, MD, USA), detached using Enzyme-free dissociation buffer (GIBCO-BRL, Gaithersburg, MD, USA) and resuspended in Ham's F12 medium (with 0.3 mM CaCl₂, 0.1% fetal bovine serum, 25 mM HEPES, pH7.3). The cell suspension was centrifuged at 300g for 5 min. The supernatant was removed, and the pellet was then resuspended in 10 mL ECB. The cell density was determined by counting with a hemacytometer and adjusted to 500,000 cells/ml in ECB.

Neuromedin U was diluted in ECB (as modified above) using 5-fold serial dilutions, and aliquoted into assay plates in triplicates at 0.1 ml/well. The cell suspension was injected at 0.1 ml/well, read and integrated for a total of 20 seconds using Dynex MLX luminometer (Dynex Technologies, Middlesex, UK). Data were analyzed using the software GraphPad Prism Version 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

GHSR-R-expressing cells showed a strong, dose-dependent response to both rat NMU-23 and porcine NMU-8 (Fig. 5). The EC₅₀ of rat NMU-23 and porcine NMU-8 in these cells is 14.1 nM and 2.1 nM, respectively, suggesting both peptides are likely endogenous ligands of GHSR-R. Untransfected cells showed no response to either form of NMU at the highest concentration tested (Fig.5). Cells transfected with plasmids expressing other G-protein coupled receptors cloned into the same vector did not show any response to NMU either. The results indicate that both porcine and rat NMU can activate GHSR-R with high affinity and lead to Ca²⁺ mobilization, most likely through the Gαq/G₁₁ pathway.

β-lactamase assays were carried out as described previously; Zlokarnik et al., 1998, *Science* 279:84-88. CH.3xNFAT-bla cells (Aurora Biosciences, San Diego, CA, USA) stably expressing GHSR-R were maintained at 37°C /5% CO₂ in DMEM + 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD, USA), 1 mM sodium pyruvate, 1 mM non-essential amino acid, 55 μM 2-mecaptoethanol, 250 μg/ml zeocin, 5.0 μg/ml puromycin. Cells were seeded at ~7,000 cells/well in 96-well clear bottom black-walled plates two days before assay.

The day the assay was performed, growth media was removed from the cells and replaced with 0.05 mL /well of phenol red-free Opti-MEM (GIBCO-BRL, Gaithersburg, MD, USA). Neuromedin U was diluted in Opti-MEM using 5-fold serial dilutions, and added into assay plates in triplicates at 0.05 ml/well. The plates were incubated at 37 °C /5% CO₂ for 4 hours.

CCF2AM (Aurora Biosciences, San Diego, CA, USA) dye was prepared as follows: To 60 μ l of Solution B (100 mg/ml of Pluroic-F127 in DMSO containing 0.1% acetic acid), 12 μ l of Solution A (1 mM of CCF2AM in dry DMSO) was added. The mixture was mixed well and then added with vigorous agitation to 925 μ l Solution C (24% w/w PEG-400, 12 ESS (Aurora Biosciences, San Diego, CA, USA) w/v). 75 μ L of Solution D (200 mM prbenecid dissolved in 200 mM NaOH) was added and then mixed. The resulting dye mixture was loaded at 20 μ l/well onto the cells. The cells were then incubated at room temperature for one hour.

Fluorescence was measured at emission wave length of 460 nM and 535 nM using an excitation wave length of 405 nM in a Tecan SpectraFluor Plus fluorescence microplate reader (Tecan Austria, Salzburg, Austria). The ratio of fluorescence between 460 nM and 535 nM was calculated and plotted using the software GraphPad Prism Version 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

CHO-NFAT-bla cells stably expressing GHSR-R showed a strong response to NMU when screened against NMU using the β -lactamase assay. The dose response of GHSR-R in CHO-NFAT-bla cells is shown in Fig. 8. HEK293CRE-bla cells stably expressing GHSR-R were also screened against NMU using the β -lactamase assay and, too, showed a strong response to NMU. The dose response of GHSR-R in HEK293CRE-bla cells is shown in Fig. 9.

EXAMPLE 9

FLIPR Assay

COS-7 cells were transiently transfected with GHSR-R-pIRESpuromycin and control vector using lipofectamine (GIBCO-BRL). Two days after transfection, cells were detached with enzyme-free dissociation buffer and seeded into 96-well plates at \sim 15,000 cells/well. After 24 h, cells were loaded with Calcium Green-1 in the presence of 2.5 mM probenecid. After washing, the cells were treated with varying concentrations of NMU. Fluorescence output was measured by a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Inc.).

FLIPR monitors changes of intracellular Ca^{2+} concentration in real time. As shown in Fig. 6, cells transfected with GHSR-R/pIRESpuromycin showed a dose-dependent response to both rat and porcine NMU. The EC50 of rat NMU-23

and porcine NMU-8 was 55 nM and 45 nM, respectively, confirming that NMU is able to activate GHSR-R and cause Ca^{2+} mobilization.

EXAMPLE 10

5 Radio-ligand Binding Assay

Cell membrane preparation: HEK-293/aeq17 cells stably expressing human GHSR-R (3 T-175 tissue culture flasks, $\sim 30 \times 10^6$ cells) were harvested by scraping, washed once in 50 mM Tris-HCl, pH 7.4, 5 mM MgCl_2 , and centrifuged at $2000 \times g$ for 15 minutes. All procedures were conducted on ice. Cell pellets were
10 homogenized in a tissue grinder with a PTFE pestle (25 strokes). Crude cell membranes were then isolated by centrifugation of the cell lysate at $13,000 \times g$ for 30 minutes. Membrane pellets were resuspended at a protein concentration of 2.8 mg/ml in 50 mM Tris-HCl, pH 7.4, 5 mM MgCl_2 .

Binding of rat ^{125}I -neuromedin U-25 to Cell Membranes Expressing
15 human GHSR-R: Neuromedin U-25 (rat) was labeled with ^{125}I at its N-terminal tyrosine residue (Woods Assay, Portland, OR) to a specific activity of ~ 2000 Ci/mmol. The binding solution (0.5 ml in 12 x 75 mm borosilicate glass tubes) contained 4 μg GHSR-R expressing cell membrane, 0.1 nM ^{125}I -neuromedin U-25 in 25 mM Tris-HCl, pH 7.4 buffer with 2 mM EDTA, 10 mM MgCl_2 and 100 $\mu\text{g}/\text{ml}$
20 bacitracin. After incubation for 1 hour at room temperature, binding reactions were filtered through GC/C filters (Whatman; presoaked for 1 hour in 1% polyethylenimine) on a 48-well cell harvester (Brandel), washed 3 x 3 ml with ice-cold 50 mM Tris-HCl, pH 7.4 buffer with 10 mM MgCl_2 . Radioactivity on the filters was quantitated by gamma counting. Competition analysis for the binding of ^{125}I -
25 neuromedin U-25 to GHSR-R expressing cell membranes indicates the presence of a high affinity binding site with a IC_{50} of 3.3 nM for unlabeled rat neuromedin U-25. As shown in Fig. 7, rat NMU-23 displayed an IC_{50} of 3.3 nM under the conditions tested.

30

EXAMPLE 11

Decreasing Food Intake with Neuromedin U

Animals and Diet: Male rats (Charles River Sprague Dawley) weighing 250-350 g were maintained in a temperature and humidity controlled
35 facility with a 12 hour light/dark cycle (4:00AM lights on). Rats were fed standard

rodent ground chow (Purina#7012). Fresh diet was provided daily. Both food and water were supplied *ad libitum* throughout both the pre- and post-surgery periods.

5 ICV cannulation: Rats under ketamine/xylazine anesthesia, were stereotaxically implanted in the lateral ventricle with a 26G guide cannula. The guide cannula (Plastics One Roanoke, Va.) was secured with three set screws and dental cement. A dummy internal cannula was placed into the guide cannula to maintain cannula patency. Rats were individually housed in Nalgene metabolism cages and allowed to recover a minimum of seven days before injection with test compounds.

10 Acclimation: Beginning the day after surgery, each rat was acclimated to the handling and injection procedure, as well as, the feeding regimen. At approximately 10:00 AM, all rats were presented with fresh chow diet. They were allowed free access to the fresh diet for one hour in order to induce satiation. At the end of the satiation period food was weighed and each rat was handled using minimal restraint and its dummy cannula was removed. Removing the dummy cannula daily
15 ensured cannula patency and acclimated the animal to the injection procedure. The rat was then returned to its cage. Food intake was measured two hours later and the following morning. Daily handling familiarized the rats with the injection procedure and minimized associated stress, an important consideration in feeding behavior studies where stress can cause hormonal changes in the body that can effect food
20 intake. Rats were not used in any experimental protocols until their food intake returned to normal.

Lateral ventricle injections: All test substances were dissolved in artificial cerebral spinal fluid (aCSF; Harvard Apparatus, Holliston Mass.). Injections were in a volume of 400 nl. An injector was constructed consisting of a 10 μ l
25 Hamilton syringe (Hamilton Co. Reno, NV. Model No. 701) with a 33 G needle. The length of the needle was such that it would extend 1 mm below the tip of the guide cannula when inserted. The syringe was attached to a Hamilton repeater. The injector was calibrated so that two pulses of the repeater delivered 400 nl of solution. The needle was held in place for approximately 30 seconds and was then removed very
30 slowly to prevent backflow of the injected material.

Confirmation of cannula placement: Once the rats were fully recovered from surgery, guide cannula placement was confirmed by evaluating the food intake induced by the injection of 5 μ g of hNPY (Peninsula Laboratories, Belmont, Calif.) after a one hour satiation period. Two hour post-injection food
35 intake was recorded for each rat. The guide cannula was considered to be in the

correct location if the NPY-induced two hour food intake was at least twice that of the two hour food intake recorded on the previous non-injection day. Rats were allowed a minimum of 72 hours between NPY injection and any test compound injection. Only NPY responsive rats were used in the experimental protocol. Cannula patency was reconfirmed after each test injection.

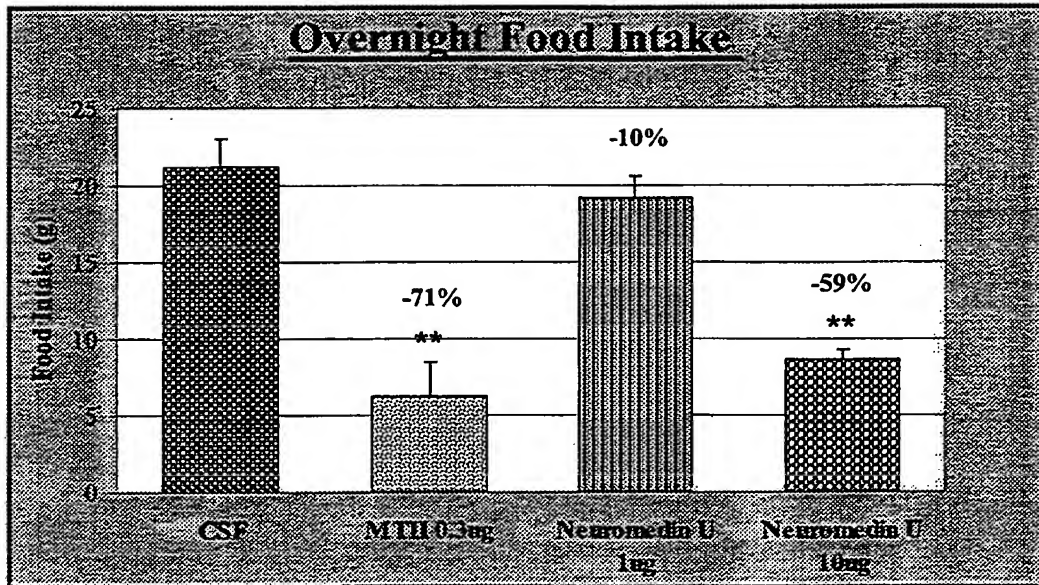
Test Compounds: NPY responsive rats were first injected ICV with aCSF to determine basal overnight food intake. To assess the possible role of the GHSR-R receptor in controlling spontaneous food intake, rats (n=6) were injected ICV with 1 or 10 μ g of the ligand peptide NMU (rat, Phoenix Pharmaceuticals, Mountainview,CA). Additional rats were injected ICV with 0.3 μ g of the peptide melanocortin agonist MTII (Peninsula Laboratories, Belmont, Calif.) as a positive control; see Murphy *et al.*, 1998 *Neuropeptides* 32:491-497.

Data analysis: Food intake measurements were taken on individual rats at different times and under different treatment regimens using a cross-over study design. In this paradigm, each rat received a vehicle dose, as well as, one or more test doses. Food consumption of treated rats was compared to aCSF vehicle treated rats using an unpaired two-tailed t-test. Percent changes in food intake were calculated relative to the aCSF vehicle from each animal. Group comparisons were made using an unpaired two-tailed t-test.

Results: The 10 μ g dose of NMU produced a 59% ($P<0.01$) suppression of food intake; see figure below.

Fig. 1 - The Effect of NMU (1 and 10 μ g) administered ICV (LV) on Overnight Food Intake in lean CRL rats on ground (Purina #7012) rodent diet

5



Conclusions: The peptide ligand NMU of GHSR-R had a marked effect on rat food intake suggesting a possible involvement of this receptor in normal feeding behavior.

10

WHAT IS CLAIMED:

1. A receptor which is at least 90% homologous to the sequence set forth in SEQ ID NO:2, free from receptor-associated proteins.
- 5 2. A receptor which is at least 90% homologous to the sequence set forth in SEQ ID NO:4, free from receptor-associated proteins.
3. A receptor comprising SEQ ID NO:2.
4. A receptor comprising SEQ ID NO:4.
5. Nucleic acid encoding a receptor of the sequence set forth in SEQ
10 ID NO:2, said nucleic acid being free from associated nucleic acids.
6. Nucleic acid encoding a receptor of the sequence set forth in SEQ ID NO:4, said nucleic acid being free from associated nucleic acids.
7. Nucleic acid which hybridizes to the sequence set forth in SEQ ID NO:1 under highly stringent conditions, said nucleic acid being free from associated
15 nucleic acids.
8. Nucleic acid which hybridizes to the sequence set forth in SEQ ID NO:3 under highly stringent conditions, said nucleic acid being free from associated nucleic acids.
9. Nucleic acid comprising SEQ ID NO:1, said nucleic acid being free
20 from associated nucleic acids.
10. Nucleic acid comprising SEQ ID NO:3, said nucleic acid being free from associated nucleic acids.
11. A vector comprising the nucleic acid of claim 9.
12. A vector comprising the nucleic acid of claim 10.
- 25 13. A host cell comprising the vector of claim 11.
14. A host cell comprising the vector of claim 12.
15. A method of identifying ligands which comprises:
 - (a) contacting cells expressing the receptor of claim 3 with a compound suspected of being a ligand specific for said receptor; and
 - 30 (b) determining whether binding occurs, binding constituting a positive indication of the presence of a ligand.

16. A method of identifying ligands which comprises:

- (a) contacting cells expressing the receptor of claim 4 with a compound suspected of being a ligand specific for said receptor; and
(b) determining whether binding occurs, binding constituting a positive indication of the presence of a ligand.

17. A method of identifying ligands for GHSR-R which comprises:

- (a) contacting cells expressing the receptor of claim 3 with a compound suspected of being a ligand specific for said receptor in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} mobilization, and
(b) monitoring for luminescence or other signal from the reporter indicating activation of the receptor, activation constituting a positive indication of the presence of a ligand.

18. A method of identifying ligands for GHSR-R which comprises:

- (a) contacting cells expressing the receptor of claim 4 with a compound suspected of being a ligand specific for said receptor in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} mobilization, and
(b) monitoring for luminescence or other signal from the reporter indicating activation of the receptor, activation constituting a positive indication of the presence of a ligand.

19. A method for determining whether a substance is a potential agonist or antagonist of GHSR-R comprising:

- (a) contacting cells expressing the receptor of claim 3 with labeled neuromedin U in the presence and in the absence of the substance; and
(b) measuring the binding of neuromedin U to GHSR-R; where if the amount of binding of neuromedin U is less or more in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GHSR-R.

20. A method for determining whether a substance is a potential agonist or antagonist of GHSR-R comprising:

- (a) contacting cells expressing the receptor of claim 4 with labeled neuromedin U in the presence and in the absence of the substance; and

(b) measuring the binding of neuromedin U to GHSR-R; where if the amount of binding of neuromedin U is less or more in the presence of the substance than in the absence of the substance, then the substance is a potential agonist or antagonist of GHSR-R.

5 21. A method of identifying ligands for GHSR-R which comprises contacting cells expressing the receptor of claim 3 with a compound suspected of being a ligand specific for said receptor, and monitoring for changes in concentration of intracellular cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of the presence of a ligand.

10 22. A method of identifying ligands for GHSR-R which comprises contacting cells expressing the receptor of claim 4 with a compound suspected of being a ligand specific for said receptor, and monitoring for changes in concentration of intracellular cyclic AMP (cAMP); an increase in cAMP constituting a positive indication of the presence of a ligand.

15 23. A method of determining whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the receptor of claim 3 with the substance in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the receptor; activation constituting a positive indication of
20 the presence of an agonist.

 24. A method of determining whether a substance is a potential agonist of GHSR-R which comprises contacting cells expressing the receptor of claim 4 with the substance in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter
25 indicating activation of the receptor; activation constituting a positive indication of the presence of an agonist.

 25. A method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting cells expressing the receptor of claim 3 first with the substance and then with neuromedin U in the presence of jelly
30 fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the receptor;

where if the amount of luminescence or signal is less in the presence of the substance than in the absence of the substance, then the substance is a potential antagonist of GHSR-R.

26. A method of determining whether a substance is a potential
5 antagonist of GHSR-R which comprises contacting cells expressing the receptor of claim 4 first with the substance and then with neuromedin U in the presence of jelly fish aequorin or other suitable reporter responsive to Ca^{2+} , and monitoring for luminescence or other signal from the reporter indicating activation of the receptor;
10 where if the amount of luminescence or signal is less in the presence of the substance than in the absence of the substance, then the substance is a potential antagonist of GHSR-R.

27. A method of determining whether a substance is a potential
agonist of GHSR-R which comprises contacting cells expressing the receptor of claim 3 with the substance, and monitoring for changes in cyclic AMP (cAMP); an increase
15 in cAMP constituting a positive indication of an agonist.

28. A method of determining whether a substance is a potential agonist
of GHSR-R which comprises contacting cells expressing the receptor of claim 4 with the substance, and monitoring for changes in cyclic AMP (cAMP); an increase in
cAMP constituting a positive indication of an agonist.

20 29. A method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting cells expressing the receptor of claim 3 with the substance, and monitoring for changes in cyclic AMP (cAMP); a marginal to no increase in cAMP constituting a positive indication of an antagonist.

25 30. A method of determining whether a substance is a potential antagonist of GHSR-R which comprises contacting cells expressing the receptor of claim 4 with the substance, and monitoring for changes in cyclic AMP (cAMP); a marginal to no increase in cAMP constituting a positive indication of an antagonist.

31. A method for the treatment or prevention of obesity which
comprises administering to a mammal in need of such treatment or prevention an
30 effective amount of neuromedin U or a GHSR-R agonist.

32. A method of decreasing food intake of a mammal which comprises administering to said mammal an effective amount of neuromedin U or a GHSR-R agonist.

5

1/16

-308 ACCTGGCTGGCTCAGCTTCCTTGGGTTAAAGCTGGGCACCTACCAGTCCCGCCCAATTTTATTATTTCACGG -229
 -228 TTTCACCTCCGAACTACTCTAGTTCAATGCCACTTTAGTGGTGGCAGATGCTTCTTCCAGTGGCATGTGACTAAT -149
 -148 CAGTCTCTACAGTGTGATACTTAGGACAGAGCTGGGATTACCTAAATGACATCTGGGTATCTCCCGCTTCTATCTAC -69
 -68 AACTGCTCCCTGGCTCAATTGTTCCATCTTTCCTGAGCGGCTCTCCCAAAATGCTTCAAGGAGCCCC -1

+ 1 CTG GTC TGC AAT ATC AGT GAG TTC AAG TGG CCC TAT CAA CCT GAG GAT CTG AAC CTT ACC 60
 1 L V C N I S E F K W P Y Q P E D L N L T 20
 TM-1—
 61 GAT GAG GCC CTG AGG CTG AAG TAT TTG GGG CCA CAG CAG ATG AAA CAG TTT GTC CCC ATC 120
 21 D E A L R L K Y L G P Q Q M K Q F V P I 40

121 TGT GTC ACG TAC CTG CTG ATC TTC GTG GTG GGC ACT CTG GGC AAC GGG CTG ACC TGC ACC 180
 41 C V T Y L L I F V V G T L G N G L T C T 60
 TM-2—
 181 GTC ATC CTG CGC AAC AAG ACT ATG CGC ACG CCC ACC AAC TTC TAC CTC TTC AGC CTC GCT 240
 61 V I L R N K T M R T P T N F Y L F S L A 80

241 GTC TCC GAT ATG CTG CTG CTC GTG GGC TTG CCT CTG GAG CTT TAT GAG ATG CAG CAA 300
 81 V S D M L V L L V G L P L E L Y E M Q Q 100
 TM-3—
 301 AAT TAC CCG TTC CAG CTG GGT GCG AGT GCG TGC TAC TTC CGA ATA CTG CTC TTA GAG ACC 360
 101 N Y P F Q L G A S A C Y F R I L L L E T 120

361 GTC TGC CTA GCT TCA GTG CTC AAT GTC ACA GCC CTG AGT GTG GAG CGT TAT GTG GCC GTG 420
 121 V C L A S V L N V T A L S V E R Y V A V 140
 TM-4—
 421 GTC CGC CCA CTC CAA GCC AAG TCT GTG ATG ACA CGG GCC CAT GTG CGC CGC ATG GTG GGG 480
 141 V R P L Q A K S V M T R A H V R R M V G 160

FIG. 1A

2/16

481 GCC ATC TGG GTC CTC GCT ACT CTC TTC TCT CTG CCC AAC ACC AGC CTG CAT GGC CTC AGT 540
 161 A I W V L A T L F S L P N T S L H G L S 180
 541 CAA CTA ACT GTG CCC TGC CGG GGG CGG CTG CCC GAC TCA CCT ATA TGT TCG CTG GTG GGT 600
 181 Q L T V P C R G P V P D S A I C S L V G 200
 TM-5
 601 CCC ATG GAC TTC TAC AAG TTG CTG GTA CTG ACT ACC GCA CTG CTC TTC TGT CTG CCC 660
 201 P M D F Y K L V V L T T A L L F F C L P 220
 661 ATG CTC ACC ATC AGT GTG CTG TAT CTG CTC ATT GGG CTG CGG CTG CGG AGG CAG AGG ATG 720
 221 M V T I S V L Y L L I G L R L R E R M 240
 721 TTG CTC CAA GTG GAG GTC AAG GGC AGG AAA ACC GCA GCA ACC CAG GAG ACC TCC CAC AGA 780
 241 L L Q V E V K G R K T A A T Q E T S H R 260
 TM-6
 781 AGG ATT CAG CTG CAA GAT AGG GGA CGG AGA CAG GTG ACC AAG ATG CTG TTT GCA CTG GTT 840
 261 R I Q L Q D R G R R Q V T K M L F A L V 280
 841 GTG GTA TTC GGC ATC TGC TGG GCT CCA TTC CAT GCT GAC CGT ATC ATG TGG AGC CTG CTG 900
 281 V V F G I C W A P F H A D R I M W S L V 300
 TM-7
 901 TAT GGA CAC TCA ACG GAA GGC CTG CAC CTG GGC TAC CAG TGT GTG CAC ATT GCC TCT GGC 960
 301 Y G H S T E G L H L A Y Q C V H I A S G 320
 961 ATC TTC TTC TAT CTC GGC TCA GCA GCC AAC CCG GTG CTC TAC AGC CTC ATG TCT ACT CGC 1020
 321 I F F Y L G S A A N P V L Y S L M S T R 340

FIG. 1B

3/16

1021 TTC CGA GAG ACC TTC CTG CAA GCC CTG GGC CTT GGA ACC CAG TGC TGT CAT CGC CGC CAA 1080
341 F R E T F L Q A L G L G T Q C C H R R Q 360

1081 CCC TAT CAT GGC TCC CAT AAC CAC ATC AGG TTG ACC ACA GGC AGC ACC CTG TGT GAC GTG 1140
361 P Y H G S H N H I R L T T G S T L C D V 380

1141 GGC CAC AGG AAC AGC AGG GAC GAA CCT CTG GCT CTG AAT GAG GAT CCA GGC TGT CAG CAA 1200
381 G H R N S R D E P L A V N E D P G C Q Q 400

1201 GAG ACA GAC CCC TCC TGA 1218
401 E T D P S * 406

FIG. 1C

4/16

1 ATG GCT TGC AAT GGC AGT GCG GCC AGG GGG CAC TT^{TM-1} GAC CCT GAG GAC TTG AAC CTG ACT 60
 1 M A C N G S A A R G H F D P E D L N L T 20
 61 GAC GAG GCA CTG AGA CTC AAG TAC CTG GCG CCC CAG CAG ACA GAG CTG TTC ATG CCC ATC 120
 21 D E A L R L K Y L G P Q Q T E L F M P I 40
 121 TGT GCC ACA TAC CTG ATC TTC GTG GTG GGC GCT GTG GGC AAT GGC CTG ACC TGT CTG 180
 41 C A T Y L L I F V V G A V G N G L T C L 60
 181 GTC ATC CTG CCG CAC AAG GCC ATG CCG AGC CCT AC^{TM-2} AAC TAC CTC TTC AGC CTG GCC 240
 61 V I L R H K A M R T P T N Y Y L F S L A 80
 241 GTC TGC GAC CTG CTG CTG CTG GGC CTG CC^{TM-3} CTG GAG CTC TAT GAG ATG TGG CAC 300
 81 V S D L L V L L V G L P L E L Y E M W H 100
 301 AAC TAC CCC TTC CTG CTG GCG GTT GGT GGC TGC TAT TTC CCG AGC CTA CTG TTT GAG ATG 360
 101 N Y P F L L G V G G C Y F R T L L F E M 120
 361 GTC TGC CTG GCC TCA GTG CTC AAC GTC ACT GCC CTG AGC GTG GAA CCG TAT GTG GCC GTG 420
 121 V C L A S V L N V T A L S V E R Y V A V 140
 421 GTG CAC CCA CTC CAG GCC AGG TCC ATG GTG ACG CCG GCC CAT GTG CCG CCA GTG CTT GCG 480
 141 V H P L Q A R S M V T R A H V R R V L C 160
 481 GCC GTC TGG GGT CTT GCC ATG CTC TGC TCC CTG CCC AAC ACC AGC CTG CAC GCC ATC CCG 540
 161 A V W G L A M L C S L P N T S L H G I R 180

FIG. 2A

5/16

541 CAG CTG CAC GTG CCC TGC CGG GGC CCA GTG CCA GAC TCA GCT GTT TGC ATG CTG GTG CGC 600
 181 Q L H V P C R G P V P D S A V C M L V R 200
 TM-5
 601 CCA CGG GCC CTC TAC AAC ATG GTA GTG CAG ACC ACC GCG CTG CTC TTG TTC TGC CTG CCC 660
 201 P R A L Y N M V V Q T T A L L F F C L P 220
 661 ATG GCC ATC ATG AGC GTG CTC TAC CTG CTC ATT GGG CTG CGA CTG CGG CGG GAG AGG CTG 720
 221 M A I M S V L Y L I G L R L R R E R L 240
 721 CTG CTC ATG CAG GAG GCC AAG GGC AGG GCC TCT GCA GCA GCC AGG TCC AGA TAC ACC TGC 780
 241 L L M Q E A K G R G S A A A R S R Y T C 260
 TM-6
 781 AGG CTC CAG CAC GAT CGG GGC CGG AGA CAA GTG ACC AAG ATG CTG TTT CTC CTG GTG 840
 261 R L Q Q H D R G R Q V T K M L F V L V 280
 841 GTG GTG TTT GGC ATC TGC TGG GCC CGG TTC CAC GCC GAC CGG GTC ATG TGC AGC CTC GTG 900
 281 V V F G I C W A P F H A D R V M W S V V 300
 TM-7
 901 TCA CAG TGG ACA GAT GGC CTG CAC CTG GCC TTC CAG CAC GTG CAC GTG ATC TCC GGC ATC 960
 301 S Q W T D G L H L A F Q H V H V I S G I 320
 961 TTC TTC TAC CTG GGC TCG GGC GCC AAC CCC GTG CTC TAT AGC CTC ATG TCC AGC CGC TTC 1020
 321 F F Y L G S A A N P V L Y S L M S S R F 340
 1021 CGA GAG ACC TTC CAG GAG GCC CTG TGC CTC GGG GCC TGC TGC CAT CGC CTC AGA CCC CGC 1080
 341 R E T F Q E A L C L G A C C H R L R P R 360

FIG. 2B

1081	CAC	AGC	TCC	CAC	AGC	CTC	AGC	AGG	ATG	ACC	ACA	GGC	AGC	ACC	CTG	TGT	GAT	GTG	GGC	TCC	1140
361	H	S	S	H	S	L	S	R	M	T	T	G	S	T	L	C	D	V	G	S	380
1141	CTG	GGC	AGC	TGG	GTC	CAC	CCC	CTG	GCT	GGG	AAC	GAT	GGC	CCA	GAG	GCG	CAG	CAA	GAG	ACC	1200
381	L	G	S	W	V	H	P	L	A	G	N	D	G	P	E	A	Q	Q	E	T	400
1201	GAT	CCA	TCC	TGA																	1212
401	D	P	S	*																	404

FIG. 2C

7/16

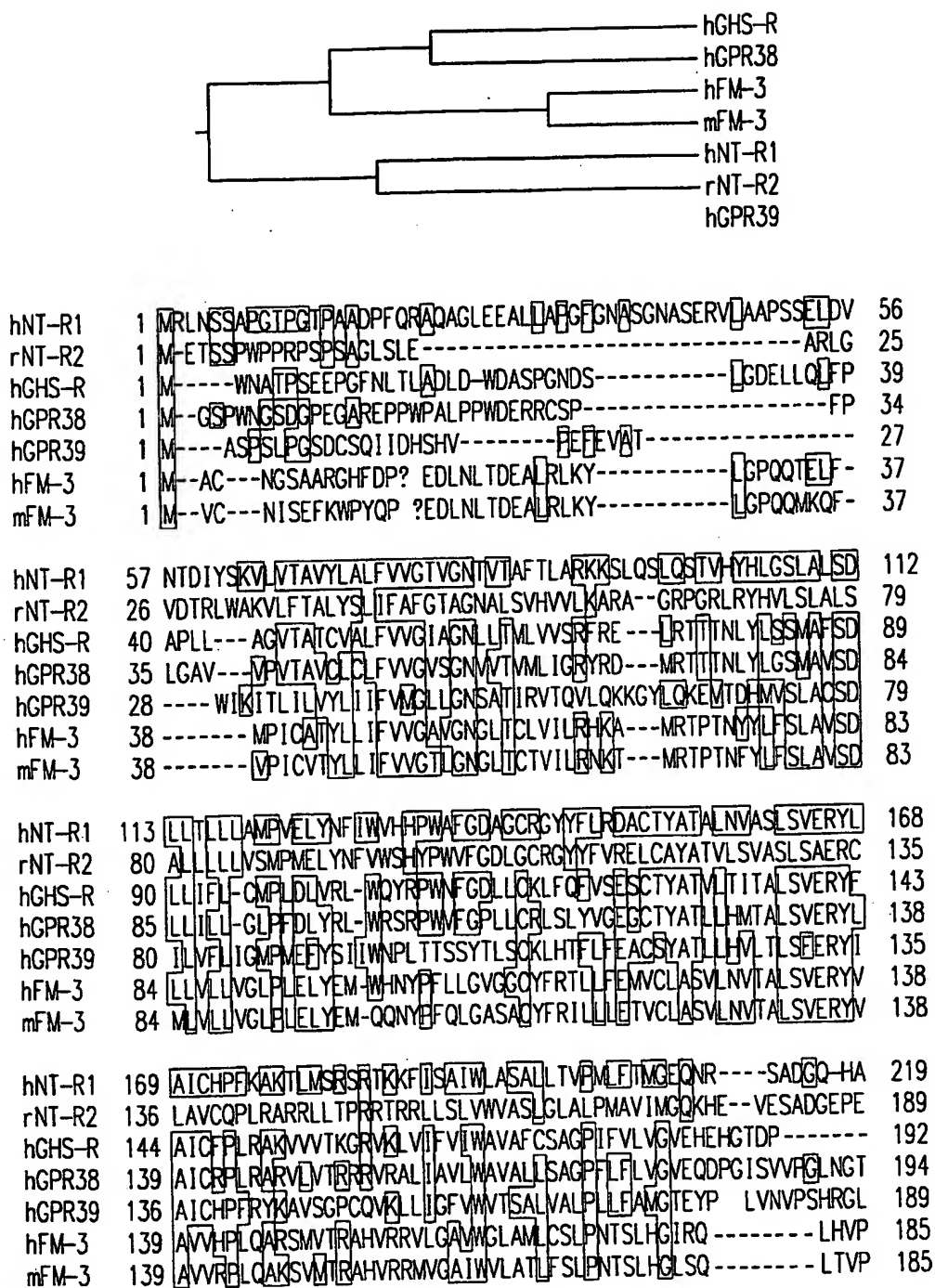


FIG. 3A

8/16

hNT-R1	220	GGLVCTPTITATVKVMIQVNTMFIFMVVISVNTIANKLTVMVRQAAEQG-	274
rNT-R2	190	PASRVCTVLVSRATLQFIQVNVLSFALPLALTAFNLGIITVNHLMALYSQVPSAS	245
hGHS-R	193	-----WDTN-----ECRPTIEFAVRSGLLTV	212
hGPR38	195	ARIASSPLASSPPLWLSRAPPPSPSG-ETAEAAALFSRECRPS--PAQLGALRV	247
hGPR39	190	TCNRSSTRHEQPETSNMSICTNLSSRWTFQSSIFGAFVY-LVLLS-----VA	239
hFM-3	186	CR-----G-PVPDSAVCMVR--PRALYNMVVQTTA	213
mFM-3	186	CR-----G-PVPDSATCSLVG--PMDFYKLVLTATA	213
hNT-R1	275	QVQTMGGE-----HSTFSAI-----EPCRVQAL---	298
rNT-R2	246	AQVSSIPSR---LELLSEEGLLGFIT---WRKTLSLGVQASLVRHKDAQIRSL--	293
hGHS-R	213	MY-WVSSIFFFLPVFCLTVLYSLIGRKLWRRRRGDAVVGAS-----	252
hGPR38	248	ML-WMTAYFFLPFLCLSILYGLIGRELWSSRRPLRGPAAS-----	287
hGPR39	240	FMDNMNQ---VLMKSQKG-----SLAGGTRPPQLRKSESEESRTA---	277
hFM-3	214	LL-----FFCLPMAIMSVLYLLIGRLRRERLLMQEAKG-RGSAAAFSRYTCRL	262
mFM-3	214	LL-----FFCLPMVTISVLYLLIGRLRRERMLLQVEVKG-RKTAATQETSHRRI	262
hNT-R1	299	-----HGVRV-LRAVVIAFVWCWLFYHVRRLMFCYISDEQWTPFIYDFYHYFM	347
rNT-R2	294	-----QHSQV-LRAIVAVYICWLPYHARRLMYCIIPDGWTNELYDFYHYFY	341
hGHS-R	253	-LRDNHKQTVKMLAVVFAFIIICWLPFHVGRYLFES-KSEFPGSLEIAQISQYCNL	306
hGPR38	288	-GREGRHQTVRVLLVWMAFIIICWLPFHVGRYIYI-NT-EDSRMY--ESQYFNI	338
hGPR39	278	-----RRQTIIFRLIMVTLAVCMFNQIRRIIAAAKPKHDWTSYFRAYMILLP	327
hFM-3	263	QQHRRQPRQVTKMLFVIMVFCICWAPFHADRMMS-VV-SQWTDGLHLAFQHVHV	316
mFM-3	263	QLQDRQPRQVTKMLFALVWVFCICWAPFHADRMMS-LVYGHSTIEGLHLAQCVHI	317
hNT-R1	348	VTNALFYVSSINPILYNLSANFRHIFLATIAG-----	381
rNT-R2	342	MVTNTLFYVSSAVTPILYNVSSSFRKLFLESLSG-----	376
hGHS-R	307	VSFVLFYLSAAINPILYNLSKKYRVAVFRL-----	338
hGPR38	339	VALQLFYLSAAINPILYNLSKKYRAAAFKL---LA-----	372
hGPR39	328	FSETFYLSVINPLLYTVSSQFRRVFQVLCORLSLQHANHEKRLRVHAHSTTD	383
hFM-3	317	ISGIFFYLGSAANPMLYSLSRFRFTFQALC--LGA-----	352
mFM-3	318	ASGIFFYLGSAANPMLYSLSRFRFTFQALC--LGTD-----	354
hNT-R1	382	-----LCPVRRRVR-----PAFSRMADSVSSNHTLSSNATRETL	417
rNT-R2	377	-----LQGEQHSVLPL-----POEAPETTSIYSFRLWGSPPNPISLGEI	415
hGHS-R	339	-----GFEPSQRKLSTLKDESSRAWTESSI	364
hGPR38	373	-----RKSRR-----GFHRSRDTAGEVAGDTGGDTMGYTETSA	406
hGPR39	384	SARFVORPLLEASRROSSARRTEKIFLSTFQSEAEPOKSKQLSLESLEPNSGAKP	439
hFM-3	353	-----CCHRLRPR-----HSS-HSLSRMTTGSTLUCDVGSLGSMWHPL	388
mFM-3	355	-----CCHRRQPY-----HGS-NHIRLTGTSTLUCDVGHNSRDEPL	390

FIG. 3B

418
417
366
413
454
404
406

hNT-R1 418 -----Y
rNT-R2 416 Q-----
hGHS-R 365 NT-----
hGPR38 407 NVKT-----MG
hGPR39 440 ANSAAENGFEHEV-
hFM-3 389 AGNDGPEAAQETDPS
mFM-3 391 AVNEDPGCCQETDPS

FIG. 3C

10/16

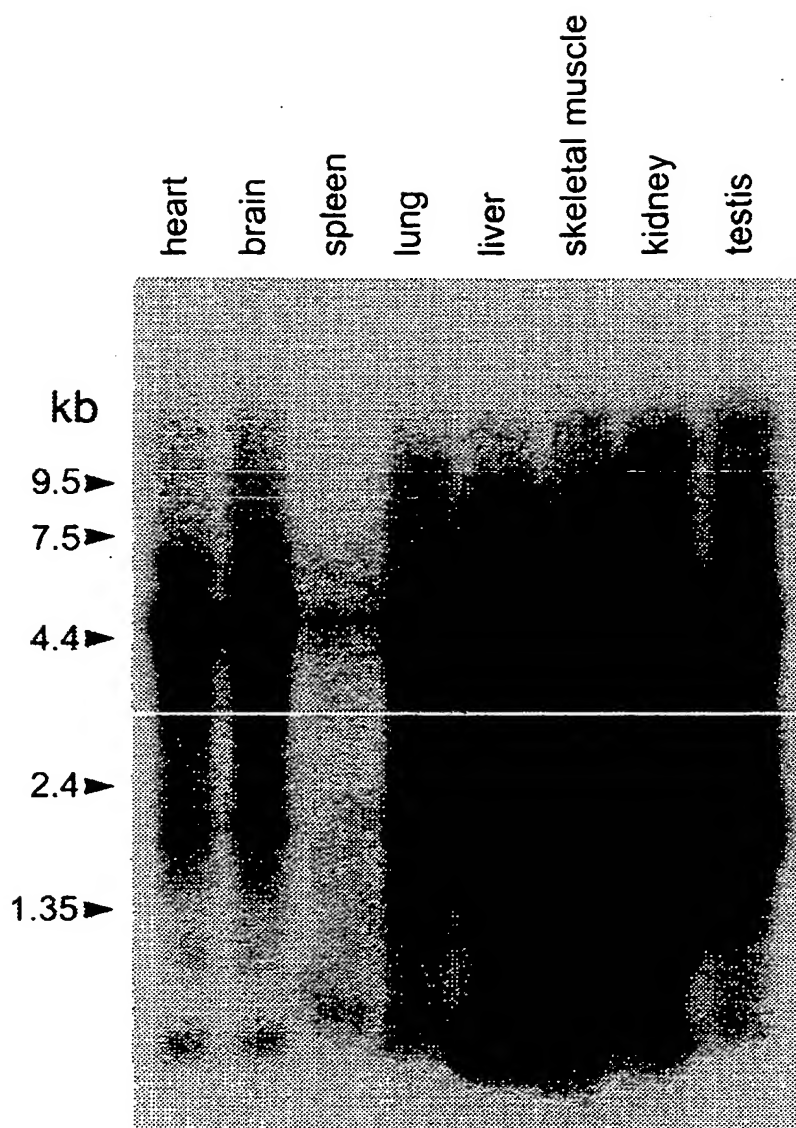


FIG.4A

11 / 16

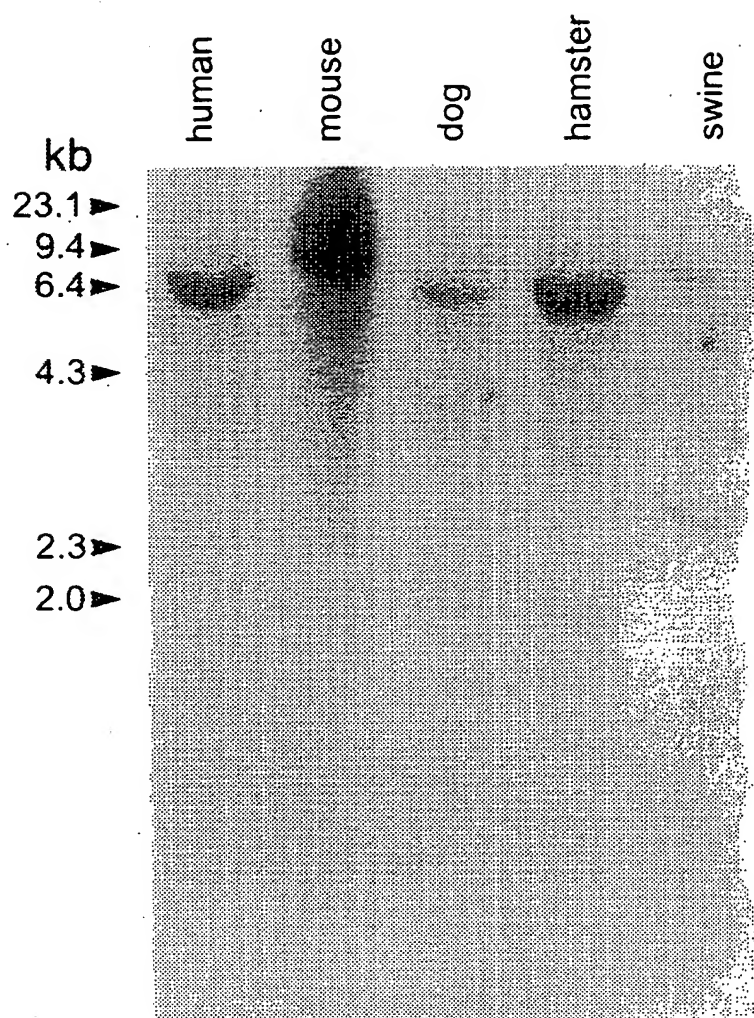


FIG.4B

12/16

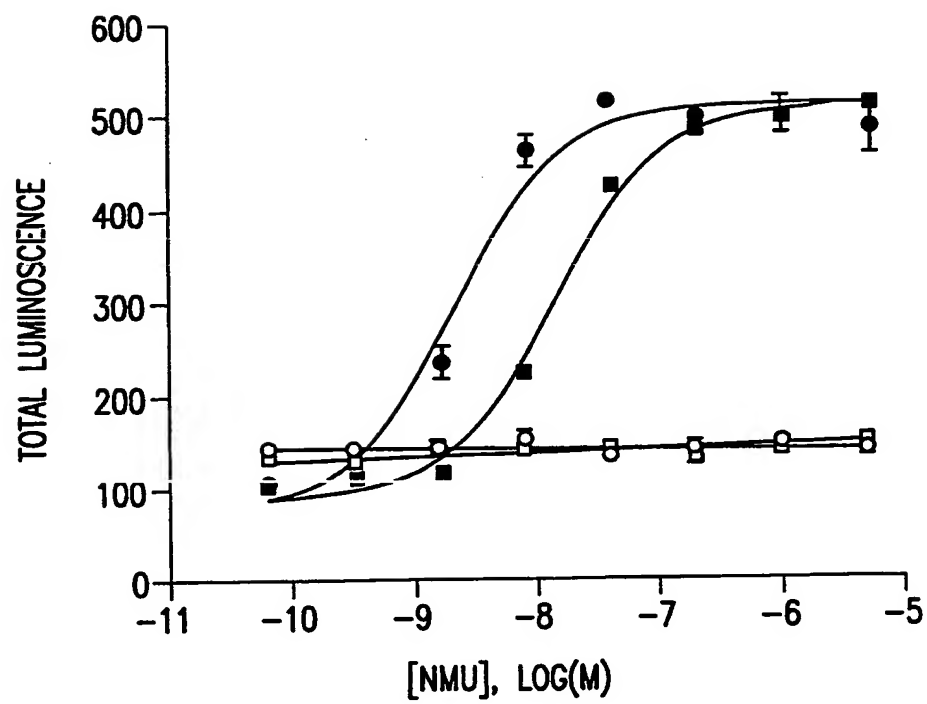


FIG.5

13/16

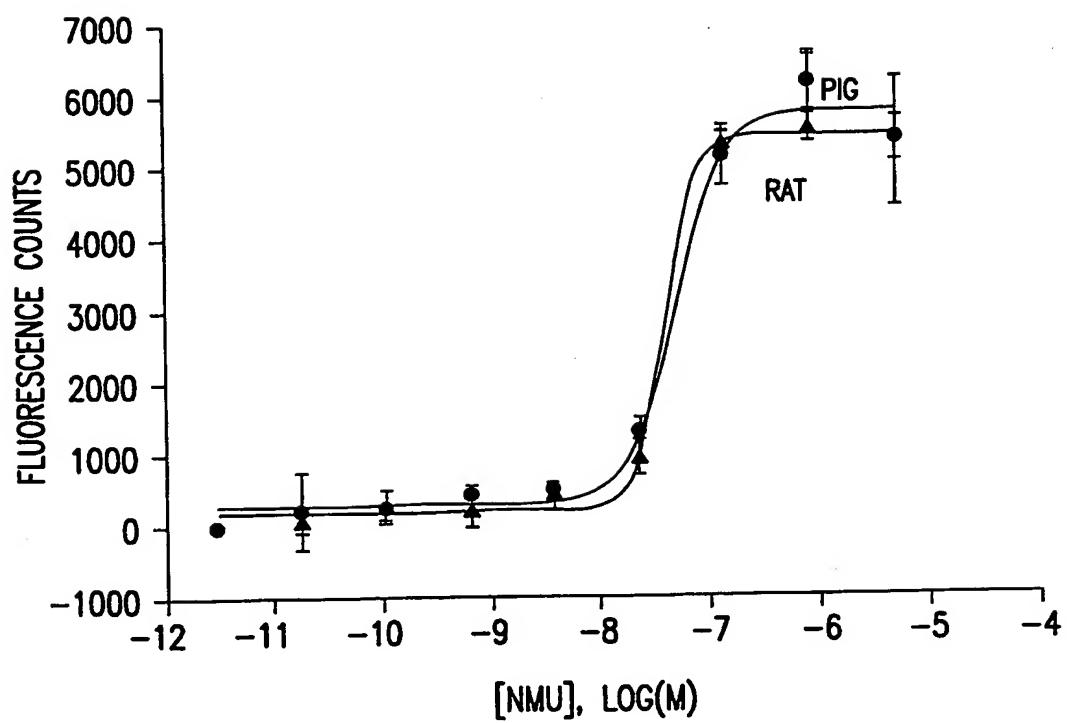


FIG.6

14/16

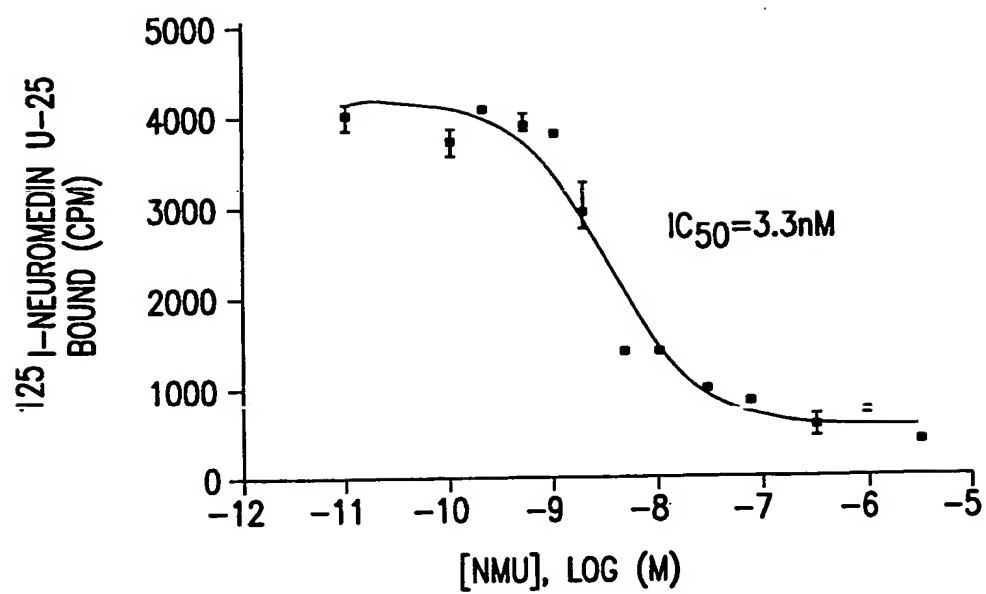


FIG.7

15/16

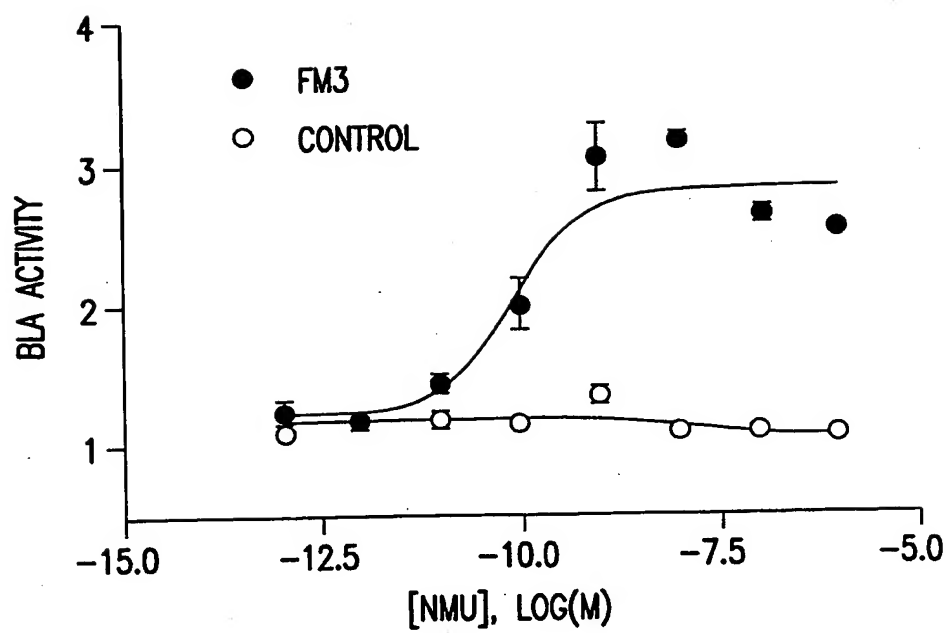


FIG.8

16/16

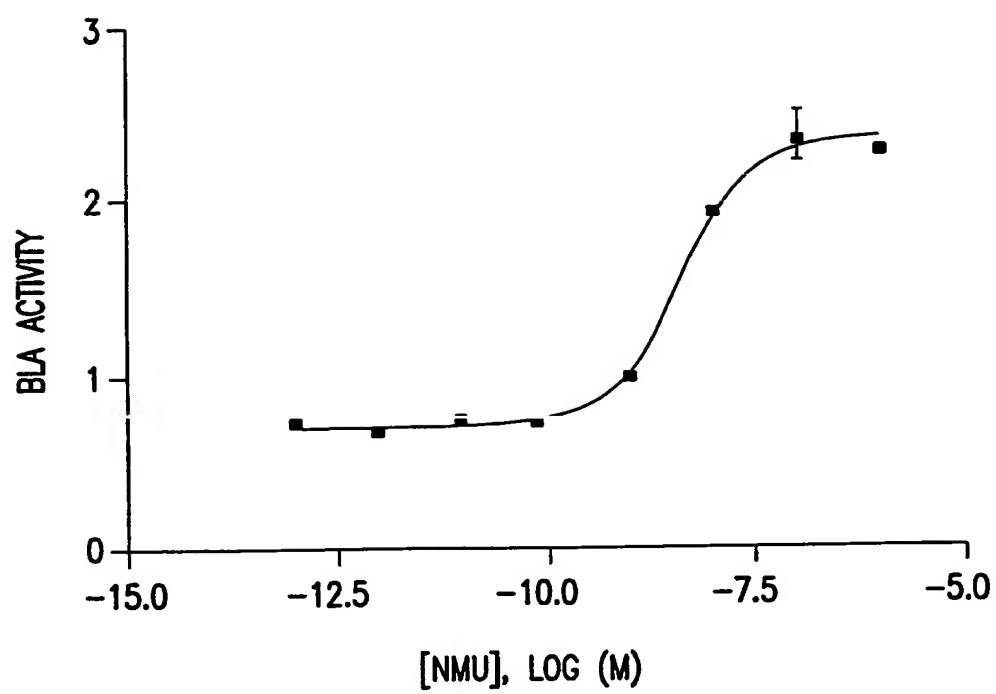


FIG.9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15941

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 530/530; 536/23.5; 435/69.1, 252.3, 325, 7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/530; 536/23.5; 435/69.1, 252.3, 325, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS US Patent full, STN via medline, embase, caplus.

Search terms: G-protein coupled receptors and growth hormone secretagogue, neuromedin U, jelly fish aequorin.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	POMES et al. Solubilization and Characterization of a Growth Hormone Secretagogue Receptor from Porcine Anterior Pituitary Membranes. Biochemical and Biophysical Research Communications. 1996, Vol. 225, No. 3, pages 939-945, see entire document.	1-4
A	GUAN et al. Distribution of mRNA Encoding the Growth Hormone Secretagogue receptor in Brain and Peripheral Tissues. Molecular Brain Research. August 1997, Vol. 48, No. 1, pages 32-29, see entire document.	1-14

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 AUGUST 1999

Date of mailing of the international search report

21 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

FOZIA HAMUD

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15941

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PONG et al. Identification of a New G-protein-Linked Receptor for Growth Hormone Secretagogues. Molecular Endocrinology. January 1996. Vol. 10, No. 1, pages 57-61, see entire document.	1-14
A	WO 98/14780 A1 (DENNIS et al.) 09 April 1998 (09/04/98), see entire document, especially claim 1 and page 7, lines 5-28.	15-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/15941

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-30
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15941

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C07K 14/705; C12N 15/12; C12N 5/10; C12N 15/63; G01N 33/566, 33/50

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

Claims 1-30 are unsearchable to the extent that they require reference to the specified sequence listing. Because Applicant has not furnished a computer readable copy of the sequence listing, no meaningful search of the sequence per se can be carried by this Authority. However, the matter of the claims has been searched to the extent possible with reference to the balance of the description.